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# *In vivo* effect of brain-derived neurotrophic factor on the survival of developing dorsal root ganglion cells

**Chaya Kalcheim, Yves-Alain Barde[1], Hans Thoenen[1] and Nicole M.Le Douarin**

Institut d'Embryologie du CNRS et du Collège de France, 49 bis Avenue de la Belle-Gabrielle, 94736 Nogent-sur-Marne Cedex, France and [1]Department of Neurochemistry, Max-Planck-Institute for Psychiatry, D-8033 Martinsried, FRG

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**Implantation of silastic membranes between neural tube and somites at somitic levels 20-24 in 30-somite-stage chick embryos results in separation of early migrated neural crest cells of the dorsal root ganglion (DRG) anlage from the neural tube and their death within a few hours [Kalcheim and Le Douarin, (1986) *Dev. Biol.*, 116, 451-460]. The *in vivo* effects of brain-derived neutrotrophic factor (BNDF) on survival of HNK-1 immunoreactive DRG cells separated from the tube were examined by implantation of laminin-treated silastic membranes (controls) or BDNF/laminin-treated membranes. In the presence of BDNF/laminin-treated membranes, 20/25 grafted embryos fixed 10 h after implantation, contained many rescued cells on the operated side. In contrast, only a few rescued cells were observed in sections on the operated in 2/11 embryos implanted with laminin-treated silastic membranes, and no rescued cells at all could be detected in embryos implanted with NGF/laminin-treated (seven embryos) or untreated silastic membranes (12 embryos). The data presented support the hypothesis that early survival and differentiation of neural crest-derived sensory cells depend on central nervous system-derived factor(s). Moreover, this is the first evidence for the *in vivo* activity of BDNF on survival of developing DRG cells.**

*Key words:* sensory ganglia/neural crest/neurotrophic factors/neuronal differentiation/chick embryo

## Introduction

Sensory ganglia of the vertebrate peripheral nervous system (PNS) are derived from both the neural crest and the ectodermal placodes (Le Douarin, 1982). The precursors of the trunk as well as cranial sensory ganglia, whose neurons originate from the neural crest, migrate and develop in close proximity to the primordium of the central nervous system (CNS), towards which the differentiating neurons send axonal projections as soon as they become postmitotic (Le Douarin, 1982, 1984).

These observations, as well as the results of back-transplantation experiments of quail sensory ganglia into the neural crest cell migratory pathways of young chick embryos, led to the hypothesis that neural crest cells developing along the sensory lineage depend for their survival and probably for their subsequent differentiation upon a CNS-derived factor (Le Douarin, 1984, 1986). This view was substantiated in experiments where deprivation of the dorsal root ganglion (DRG) anlage from contact with the CNS by neuralectomy (Teillet and Le Douarin, 1983) or by implantation of a mechanical barrier (Kalcheim and Le Douarin, 1986) (a thin silastic membrane inserted between the CNS and the DRG primordium) caused the death of DRG cells. Moreover, local implantation of such membranes pretreated with an extract of 3- and 4-day-old neural tubes, temporarily rescued significant numbers of DRG cells.

In the present study, we test the effect of brain-derived neurotrophic factor (BDNF) on the survival of developing DRG cells separated from the neural tube by a mechanical barrier. BDNF, a small basic protein isolated from the brain (Barde *et al.*, 1982), has been purified using its ability to selectively support the survival of sensory neurons. It is known to act early in the development on sensory neurons, provided laminin is used as a substrate (Lindsay *et al.*, 1985), and has been hypothesized to reach the developing sensory neurons by their central processes (Lindsay *et al.*, 1985; Davies *et al.*, 1986a). We show here that BDNF, adsorbed along with laminin on silastic membranes rescues early DRG cells separated from the neural tube.

## Results

As reported earlier (Kalcheim and Le Douarin, 1986), the implantation at the 39-somite-stage of a 20-50 μm silastic membrane between neural tube and somites at somitic levels 20-24 leads to the rapid death of the early migrated neural crest cells forming the DRG anlage (Figure 1a and Table I).

Laminin-BDNF coated membranes were then implanted. In 20/25 (80%) successfully grafted embryos with these membranes, sacrificed 10 h after implanation, neural crest cells were obtained in a distal position with respect to the neural tube (Figure 1b). Thirty hours after grafting the membranes incubated in 100 ng/ml BDNF, rescued cells were present in 2/8 embryos (24%) (Table I). A similar success rate (2/11 embryos with rescued cells) was obtained 24-35 h after implantation of membranes pretreated with 1 μg/ml BDNF. Neural crest-derived cells were visualized with HNK-1 monoclonal antibody, known to stain an important subpopulation of migrating crest cells and their derivatives (Abbo and Balch, 1981; Tucker *et al.*, 1984).

In an attempt to quantify the extent of survival of HNK-1 positive cells, we counted the number of consecutive sections containing rescued cells, as compared to the number of sections containing a DRG in the unoperated, contralateral side. In 20 BDNF-implanted embryos, almost 66% of the sections with a DRG in the normal side contained rescued cells in the operated area, as evidenced by their HNK-1 immunoreactivity, when processed 10 h after implantation (Table II). In 14 of these embyros, the percentage of cells per section (compared to the normal side) ranged between 20 and 50% and between 5 and 20% in the other six successfully grafted embryos. In embryos where untreated membranes were implanted, no HNK-1 positive cells could be found distal to the membranes (Table I). Like *in vitro* (Lindsay *et al.*, 1985), interestingly, the presence of laminin seems to be necessary to see an effect of BDNF. Indeed, no significant effect of BDNF could be seen when adsorbed on collagen-treated membranes (Table I). Laminin alone had a modest effect on crest cell survival in two out of eleven embryos (Table I). NGF, ad-

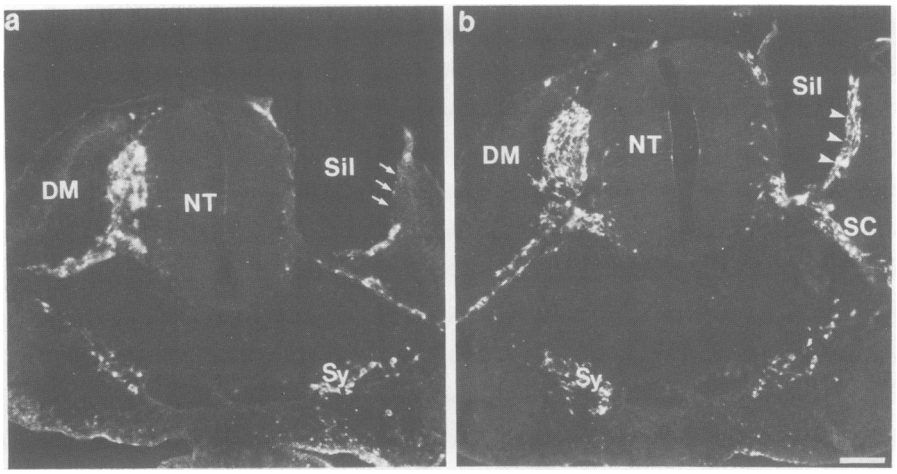


**Fig. 1.** Effect of BNDF on the survival of HNK-1 immunoreactive neural crest cells of the DRG anlage. Both photomicrographs represent transverse sections through the anterior somitic regions of 40-43 somite-stage chick embryos implanted with (**a**) untreated silastic membrane (section through somite 21) and (**b**) BDNF-laminin-treated silastic membrane (somite 21). Note in (b) the presence of many HNK-1 positive crest-derived cells in a distal position with respect to the implant (arrowheads). In contrast in (a) no fluorescent cells are detected in the same position (arrows) whereas DRG cells remaining in contact with the neural tube, Schwann cells lining the ventral root fibres (SC) and the primary sympathetic chain (Sy) were not affected. DM, dermomyotome; NT, neural tube; Sil, silastic membrane.

**Table I.** Number of embryos with rescued (HNK-1 positive) neural crest-derived cells of the DRG anlage in all experimental series

| Treatment | Embryos sacrificed (hour after grafting) | |
|---|---|---|
| | 10 h | 30–35 h |
| Untreated silastic membrane | 0/12 | 0/5 |
| Laminin-treated silastic membrane | 2/11 | 0/8 |
| BDNF on collagen-treated silastic membrane | 1/7 | 0/14 |
| BDNF on laminin-treated silastic membrane | 20/25 | 2/8 |
| NGF on laminin-treated silastic membrane | 0/7 | 0/6 |

Silastic membranes were treated as described in the legend of Figure 1.

sorbed like BDNF on laminin-coated membranes, was inactive (Table I).

## Discussion

In the present paper we demonstrate the *in vivo* effect of a known CNS-derived factor, BDNF, on survival of migrated neural crest cells comprising the DRG anlage. BDNF, adsorbed on laminin-coated silastic membranes rescued temporarily an important population of developing DRG cells separated from the neural tube. Under the same conditions, NGF was without activity; this finding demonstrates that these early DRG cells are responsive to BDNF before NGF. A simple explanation would be that the expression of the NGF receptor occurs slightly later in development than that of the BDNF receptor. Indeed, it has been shown that early migrating quail neural crest cells do not bind [$^{125}$I]-NGF (Bernd, 1985). Also, using 4-day-old DRG explants, a response to BDNF could be observed before one to NGF (Davies *et al.*, 1986b). Taken together, these findings suggest that the expression of the receptor for neurotrophic factors is tightly linked with the time of target innervation, the first target to be reached being the CNS or DRG.

**Table II.** Quantitative estimation of the rescued HNK-1 positive cells

| Treatment | No. of embryos with rescued cells | Sections with rescued cells in operated as compared to control sides[a] (%) | Estimate of the amount of HNK-1 positive cells in operated vs. control sides[b] |
|---|---|---|---|
| BNDF on laminin-treated silastic[c] | 20 | 65.7 ± 13 | Few (in 6/20 embryos) Many (in 14/20 embryos) |
| BDNF on laminin-treated silastic[d] | 2 | 20.5 ± 4.5 | Few (in 2/2 embryos) |

[a]Values represent the mean ± SEM of the percentage of sections with rescued cells in the embryos analysed. In each embryo, serial sections corresponding to an equivalent of 2–4 DRG on the unoperated side were counted. Each normal DRG in 40-somite-stage embryos was found on ~25 transverse sections, each 5 μm thick.
[b]The number of HNK-1 positive cells located distal to the implants compared to that in the normal unoperated ganglia was estimated by weighing paper projections of pictures comprising the fluroescent areas of five sections in the same embryos. The percentage of fluorescent cells in operated vs. control sides was so estimated to vary between 5 and 50%: 5–20% was designated as 'few' and 20–50% as 'many'.
[c]Embryos sacrificed 10 h after grafting.
[d]The only two embryos with surviving cells found among those sacrificed 30 h after grafting.

The results presented in this study show that not only target-derived neurotrophic factors are important for the survival of early neural crest-cell derivatives, but also laminin, found *in vivo* around the neural tube and the developing DRG (Rogers *et al.*, 1986). This is in full agreement with previous *in vitro* studies (Lindsay *et al.*, 1985) and suggests that the presence of laminin represents a permissive condition for the survival action of BDNF.

Whereas the combination of laminin and BDNF allows the effect of a neural tube extract to be reproduced 10 h after implantation of the membranes, the effects at 32 h are less than those observed before with neural tube extract (Kalcheim and Le Douarin, 1986). Why this is so remains unknown. The stability of BDNF under our experimental conditions has not been deter-

mined. Unfortunately, a second application of BDNF and laminin is not feasible in this system. In addition, it is possible that components other than BDNF and laminin, present in a total neural tube extract, are required for the prolonged maintenance of the early DRG cells.

In conclusion, the data presented in this and previous studies suggest that neurons giving rise to the DRG require early in development a neurotrophic factor from their central target (the neural tube) and that this factor is BDNF. In addition, the presence of laminin seems to be also a necessary condition *in vivo* for the survival effects of BDNF.

## Materials and methods

*Preparation of silastic membranes*

Silicone rubber membranes (silastic, Dow Corning Corp., Midland, MI) were prepared as already described (Kalcheim and Le Douarin, 1986). Rectangles of silastic were cut to fit a length equivalent of 3 or 4 somites and implanted either untreated, or treated as follows: silastic membranes were incubated overnight at 4°C in a solution of rat-tail tendon collagen, rinsed for 1 h at room temperature in Tyrode buffer and transferred to the BDNF solution (see below) for 4 h at room temperature under agitation, followed by overnight incubation at 4°C. Alternatively, the membranes were incubated overnight at 4°C in a 20 μg/ml solution of laminin (Bethesda Res. Lab., MD) in Tyrode buffer followed (when indicated) by incubation with BDNF (100 ng/ml) or NGF (30 ng/ml) in the same buffer. Incubation volumes were of 0.5 ml.

*Purification of NGF and BDNF*

NGF was purified as described (Bocchini and Angeletti, 1960; Suda *et al.*, 1978). BDNF was purified as described by Lindsay *et al.* (1985) except that the hydroxylapatite eluate was made 1.0 M with solid potassium phosphate and first loaded onto a 20 ml octylsepharose column. The flow through was loaded onto and eluted from a phenylsepharose column as before. The eluate was loaded onto a reverse phase, C3 column (Beckman, Inc.) equilibrated with 0.1% TFA. BDNF was eluted with an 0–40% acetonitrile linear gradient in 0.1% at 0.5 ml/min and 1% acetonitrile per min. The biological activity corresponding to BDNF elutes at ~30% acetonitrile. The eluate was concentrated by evaporation, resuspended in $H_2O$, and stored frozen until use. BDNF appeared as one band on SDS gel migrating at the level of cytochrome *c*, as reported before (Lindsay *et al.*, 1985).

*Membrane implantation*

Chick embryos were operated at the 30–32-somite stage. The membranes were introduced into a longitudinal 3 to 4-somite-long incision performed unilaterally between the neural tube and somites at the level of somites 20–24. At these somitic levels, neural crest cells constituting the DRG anlage are accumulated in a dorsolateral position on the neural tube, mostly facing the anterior somitic half, also with some cells still located opposite the posterior somitic region (Teillet *et al.*, 1987).

*Immunofluorescence*

Embryos were sacrificed 10 and 30–35 h after grafting and the operated areas were fixed in Bouin's fluid and embedded in paraffin wax. Five micrometer serial sections were cut and fixed on gelatin-coated glass slides. Relevant sections were bathed in toluene, rehydrated and rinsed in phosphate buffered saline (PBS), pH 7.4. Slides were stained with undiluted HNK-1 hybridoma culture supernatant for 1 h at room temperature, washed twice with PBS and then incubated with fluroescein-isothyocyanate-conjugated goat-anti mouse IgM (Cappell Laboratories, Inc., Cochranville, PA) diluted 1:50 in PBS containing 0.5% bovine serum albumin. Sections were mounted and photographed under epi-illumination on Tri-X film (Kodak) at 400 ASA with a Leitz Vario Orthomat camera mounted on a Leitz (Weitzlar) Orthoplan microscope. Bar = 50 μm.

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